Naval Medical Research Institute
8901 Wisconsin Avenue
Bethesda, MD 20889-5607

NMRI 96-53 September 1996

CELL BIOLOGY OF HYPOXIA - 1996

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Naval Medical Research and
Development Command
Bethesda, Maryland 20889-5606

Bureau of Medicine and Surgery
Department of the Navy
Washington, DC 20372-5120

NOTICES

TECHNICAL REVIEW AND APPROVAL

NMRI 96-53

The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

THOMAS J. CONTRERAS
CAPT, MSC, USN
Commanding Officer
Naval Medical Research Institute

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Technical Report (Confernce)

4. TITLE AND SUBTITLE

Cell Biology of Hypoxia, 1996

5. FUNDING NUMBERS

PE - 63706N
PR - M0095
TA - 001
WU - 1532

6. AUTHOR(S)

T.B. Nielsen and J.L. Kidwell (eds)

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Naval Medical Research Institute
Commanding Officer
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5607

8. PERFORMING ORGANIZATION REPORT NUMBER

NMRI 96-53

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Naval Medical Research and Development Command
National Naval Medical Center
Building 1, Tower 12
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5606

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

DN241220

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Hypoxia resulting from hemorrhage is a major concern of military medicine, based on studies of mortality and morbidity of battle casualties. In severe hemorrhage or exsanguination, tissues and cells are effectively isolated from the systemic circulation and the response of cells to hypoxia becomes critical. A conference was held on September 9 and 10, 1996 in Gaithersburg, Maryland to evaluate the current state of the field of cell biology of hypoxia. Scientific results were presented by Drs. Li Zhang, Dean Jones, John Lemasters, Joel Weinberg, Anne Murphy, Alexander Murashov, Kurt Henle, Sudhir Shah, Gregory Gores, Robert Winn, Chung Hsu, Florence Rollwagen, Thor B. Nielsen, and Kenneth Proctor. This report provides the presentation schedule, abstracts of the presentations, a list of participants, and, in particular, a discussion of the implications of the field for military medicine, and consideration about the direction of future research.

14. SUBJECT TERMS

Mitochondrial permeability transition; hemorrhage; preconditioning; far forward casualty care; stress protein

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

DTIC QUALITY INSPECTED 3

CELL BIOLOGY OF HYPOXIA, 1996

September 9 and 10, 1996

Gaithersburg, Maryland

Organized By:
T.B. Nielsen and A. Murphy

Edited By:
T.B. Nielsen and J.L. Kidwell

Sponsored By:
Office of Naval Research

Hosted By:
Naval Medical Research Institute

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September 10, 1996

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INTRODUCTION

Dr. Anne Murphy, George Washington University Medical Center

I'm Dr. Anne Murphy. I've spoken with most of you, or many of you, over the phone over the course of the past couple months in trying to get this meeting organized. I am a researcher like most of you, who is funded by the Office of Naval Research. I do research on the effects of hypoxia on the mechanisms of damage to cells during hypoxic periods. So I'm actually one of you, but I happen to be local and, therefore, was recruited to help organize the meeting. First of all, I'd like to introduce Thor Nielsen, who has been the major organizer of this meeting. He is with the Naval Medical Research Institute in Bethesda. I'd like to introduce, as well, two of his assistants: Johanna Kidwell and Lisa Clark-Dalton.

The next thing I need to do is introduce CAPT Thomas Contreras. He's going to introduce the general session. CAPT Contreras is a physiologist by training. He's now the Commanding Officer of the Naval Medical Research Institute. His previous position was as Executive Officer of the Naval Health Research Center in San Diego, so he brings with him not only a lot of organizational skills, but a lot of scientific background as well. CAPT Contreras.

CAPT Contreras, Naval Medical Research Institute

Thank you. Good morning and welcome to the 1996 Cell Biology of Hypoxia conference. It is a great pleasure to be your host. You've come to Washington, DC at a time when most of it's under water due to Hurricane Fran, so maybe your chances of visiting some of the landmarks around the DC area maybe limited, but take an opportunity to go down and see them. I'd like to thank each and every one of you for coming to this very important conference. We need people like yourselves to guide us along in the areas that we should focus on in the future. Specifically in this area, your help is very, very useful. The area of hypoxia is an area of research that the Navy is keenly interested in. It is very crucial to our war fighting mission. The fact that the Office of Naval Research is the sponsor of this conference clearly indicates that they also agree that this is a critical area of research. We need to do more of it. Again, I welcome you all to Washington, DC. I hope that your participation in this conference will be as beneficial to you as I know it will be to the Navy. Thank you very much.

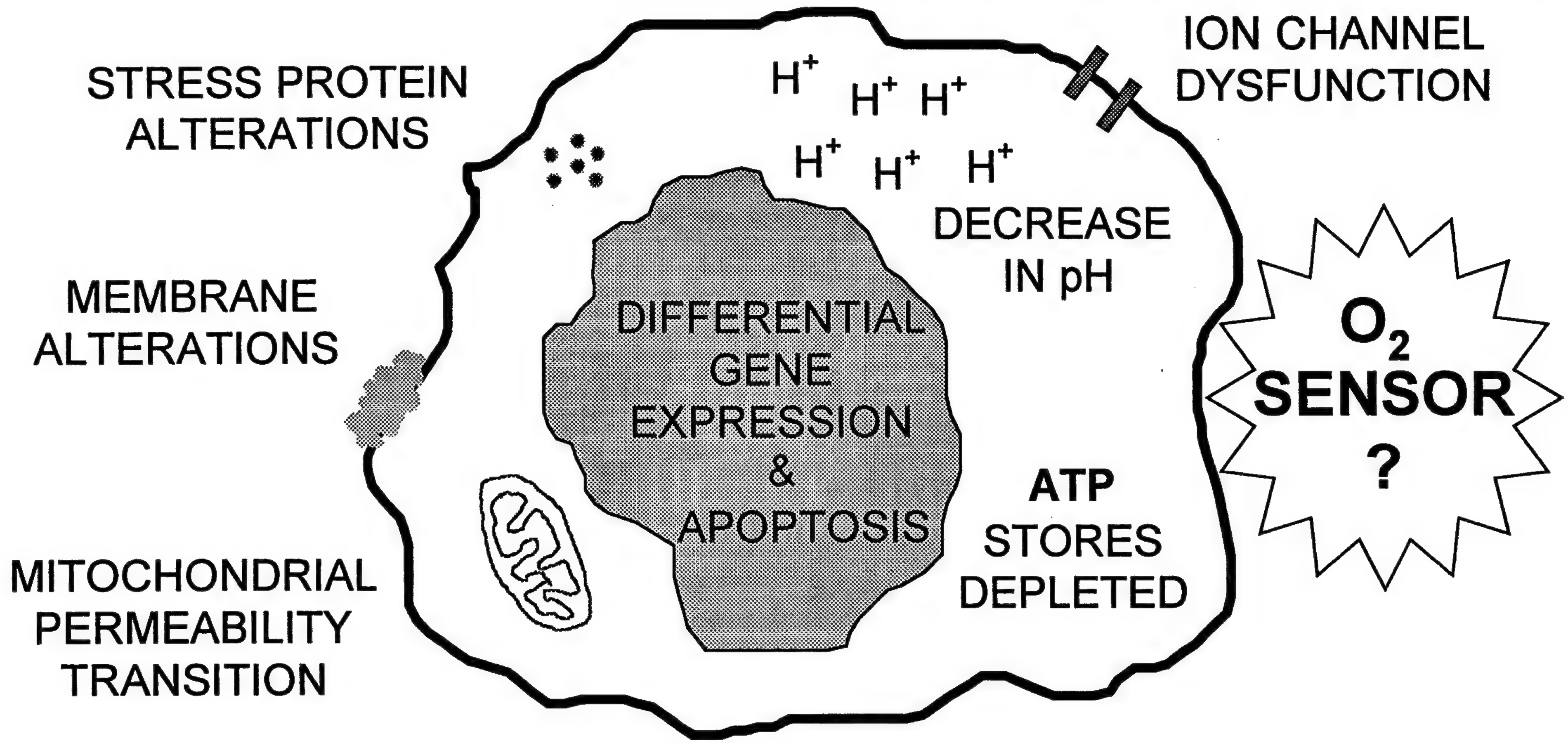
Dr. Anne Murphy

I'd like to now introduce Dr. Constance Oliver. She is in charge of biomedical science and technology at the Office of Naval Research and has been the idea person for bringing this meeting about. Many of you know her very well as the contact person of the Office of Naval Research, through which your grants have been either awarded or reviewed. She's going to give us a statement of the overall goals for this meeting and the reason that the Office of Naval Research is very interested in hypoxia. Dr. Oliver.

Dr. Constance Oliver, Office of Naval Research

Well good morning. Thank you, Anne, for your willingness to participate in this endeavor. It's a pleasure to see a lot of old friends and to have a chance to meet and talk with a number of you that so far I've only communicated with over the telephone. I want to expand a little bit on what CAPT Contreras has said, which is: why should the Navy be interested in hypoxia? To give you a little bit of a background into this program, the particular program that many of you are participants in was started in 1995. It's an outgrowth of an older program on the cell biology of trauma that was managed by Dr. Jeannine Majde, who is with us today. If we need some historical perspective, Jeannine is here to provide that as well as scientific expertise. The objective of the cell biology of hypoxia program, as I think most of you are aware, is to understand the molecular mechanisms of cellular adaption to hypoxia. The reason the Navy is interested in this particular area of research is to develop new strategies for life sustainment of combat casualties. Unlike the civilian sector that normally evacuates casualties immediately, the military does not always have this option. If you have an injured soldier or sailor on a battlefield or on shipboard who can't be evacuated, then what can we do? What mechanisms can we develop to sustain life? The basis of this program is to provide basic scientific understanding of what happens to cells when they're deprived of oxygen. Are there areas that we can exploit pharmacologically to make cells think that they're perfectly happy and in a perfectly normal environment? Without this information it will be impossible to come up with pharmacological interventions or other therapeutic strategies. This slide is a diagrammatic representation of many of the areas that will be covered in the next day and a half (Figure 1). As all of you know, hypoxia affects many if not all cellular processes. The people who are going to be speaking here today will be touching on many many different aspects of cell function including cytoskeleton membrane alterations, mitochondrial changes, ion changes within the cells, changes in gene function, gene regulation, and apoptosis. At this point we really don't know whether any or all of these areas are going to be likely targets for intervention, but the focus is to exploit what we can learn at a basic level and turn that into a therapeutic intervention. That may be to reduce cellular metabolism in such a way that cells survive the hypoxia, or to provide drugs that can treat the injury that occurs during reoxygenation. Tomorrow afternoon, the final section will be a panel discussion, and for me and for the Office of Naval Research, this is really one of the most critical portions of a meeting such as this. We've asked a number of people to participate in the panel, but we also need everyone's participation. We'd like to take that time to assess the state of the art, the state of the research. Where are we? Are there gaps? Are there areas that we need to go into? One area that I'm very interested in is identifying a cellular oxygen sensor. What we need to assess is basic research issues in a military context. Are we going in the right direction in terms of our thinking for providing pharmaceutical intervention for traumatic injury? Are there gaps in our knowledge, are there gaps in our goals, and in our targets? So I would really urge all of you, as you listen to the talks and as you give your own presentations, to think about this last hour tomorrow afternoon which is where I really would like your input and help in setting some of the future goals and the directions of the program, both scientifically and militarily. Welcome and thank you for your participation.

Hypoxia Affects Many Cellular Processes



Characterization of the Master Regulator of Oxygen Sensing and Utilization - the Heme Activating Protein 1

Li Zhang, Ph.D.
Department of Biochemistry
New York University Medical Center

Heme is central to oxygen sensing and utilization in all living organisms. Both heme and hemoproteins can act as oxygen sensors and mediate cellular responses elicited by changes of oxygen concentration or hypoxia/reoxygenation in diverse organisms. Our laboratory focuses on studying the roles of heme in oxygen sensing and utilization in eukaryotic cells. Our experimental systems include both yeast and mammalian cells. We first used the yeast *Saccharomyces cerevisiae* as a model system because it is the only eukaryote in which the master regulator of oxygen sensing and utilization, the heme-activating protein 1 (HAP1), has been identified and cloned.

In yeast, heme synthesis is directly correlated with oxygen concentrations. Heme mediates cellular responses to changes of oxygen concentrations through the transcriptional activator HAP1. We found that, in the absenceof heme, HAP1 is bound by certain cellular factors and forms a high molecular weight complex. This complex is critical for heme regulation of HAP1, and its formation requires the cooperation of three heme-regulatory domains of HAP1. Heme presumably binds to the heme-responsive motifs of HAP1, and might therefore change HAP1 conformation, causing the disassembly of the high molecular weight complex. As a result, HAP1 is free to dimerize and bind to DNA with high affinity, thereby activating transcription. Our studies on HAP1 will lay the basis for studying oxygen sensing in mammalian cells.

Integration of Sensing Mechanisms for Rapid and Chronic Hypoxia

Dr. Dean P. Jones, Ph.D.
Department of Biochemistry
Emory University School of Medicine

Aerobic organisms must respond to protect against short-term interruptions in O_2 supply and to acclimatize to chronic O_2 deficiency. Both types of responses are essential yet serve very different functions. The former are critical to tolerate traumatic injury, cerebrovascular accidents and other ischemic conditions until O_2 supply is restored while the latter are essential for an organism to function optimally at high altitudes or with pulmonary or cardiac insufficiency. The biologic strategies for these responses are opposite: tolerance to abrupt anoxia is best achieved by depression of energy requiring functions (e.g., ion transport, biosynthesis) while adjustment to chronic hypoxia involves altered expression of the molecular machinery and structural changes to optimize function. Ironically, these latter changes do not necessarily improve tolerance to anoxia but can reduce antioxidant defenses and make cells more vulnerable to ischemia/reperfusion injury. This is in direct contrast to transient exposures to ischemia which can protect tissues from subsequent ischemia "ischemic preconditioning"). The sensing mechanisms for these responses to O_2 deficiency remain poorly defined, but the distinction between anoxic tolerance and hypoxic acclimatization must be recognized as studies are performed to identify these sensing mechanism. Evolution of aerobic-tolerant anerobic microbes preceded evolution of multicellular organisms that are dependent upon O_2 for energy metabolism. Consequently, primordial sensing mechanisms probably depended upon detection of either reactive oxygen species or redox changes rather than sensing of molecular O_2 or cellular energetics. Existing O_2 sensing mechanisms therefore probably include these parameters as well as responses to O_2, ATP/ADP, and changes in membrane potentials. For direct sensing of O_2, two general mechanisms occur, one in which O_2 binding to a hemoprotein provides an initial signal and another in which the product of an O_2-dependent enzyme provides a signal. Although there are over 100 known O_2-dependent enzymes, the ones involved in heme biosynthesis are among the most interesting for O_2 sensing because they are distributed between the mitochondria and cytoplasm and have Km values that are high relative to usual cellular O_2 concentrations. Thus, the concentrations of the product of the pathway (heme) and intermediates in the pathway (coproporphyrinogen, protoporphyrinogen, protoporphyrin) can vary with O_2 concentration and serve in signal transduction. While the available evidence for the involvement of this pathway in O_2 sensing is largely focused on transcriptional activation by heme, it is of great interest that the heme intermediates are lipophilic polyvalent anions with 5-member ring systems, physical characteristics that are shared with calciphor polymers. Di-Calciphor and tri-calciphor protect against cerebral ischemia and anoxic cell injury by protecting against mitochondrial failure. Thus, heme and heme precursors, whose concentrations vary as a function of O_2 availability, may play a central role both in signaling a depression of energy metabolism to allow tolerance to abrupt anoxia as well as transcriptional regulation to allow acclimatization to chronic hypoxia.

INSIGHTS PROVIDED BY GLYCINE CYTOPROTECTION INTO CELLULAR MECHANISMS OF HYPOXIC INJURY

Joel M. Weinberg, M.D.
University of Michigan Medical Center
Ann Arbor, Michigan

During the past decade, glycine has emerged as an unexpected, major determinant of cellular susceptibility to hypoxic and related forms of acute injury in diverse cell types including kidney tubules, hepatocytes, and endothelial cells. The effect of glycine is robust. Lethal plasma membrane damage that would be maximal within 15-30 min. in the absence of glycine is completely prevented for up to several hours with full effects requiring 2-5 mM concentrations of the amino acid. These levels of glycine are likely present during most forms of *in vivo* hypoxia/ischemia, but are almost always absent during *in vitro* study conditions unless replaced. Cytoprotection by glycine does not involve its metabolism, and alanine, other specific small neutral amino acids, and compounds active at ligand-gated and other chloride channels can reproduce it to various degrees. However, a binding interaction with a specific protein target remains to be defined and the plasma membrane permeability defect blocked by glycine, although having characteristics of a size-limited pore, is far larger than a chloride channel. Glycine cytoprotection does not require amelioration of any of the classical pathophysiological mediators of hypoxic injury, but, rather, allows for maximal expression of their effects before generalized, nonspecific cell disruption and, thus, provides new perspectives into likely and possible roles for a variety of processes whose relative importance has been heretofore incompletely defined despite much work. Among such events are alterations of intracellular calcium, phospholipase activation, cytoskeletal disruption, lipid peroxidation, and the mitochondrial permeability transition. Recent new insights into each of these processes from studies using glycine will be reviewed.

Bcl-2 Inhibits Cellular Injury Induced by Transient Chemical Hypoxia/Aglycemia and Potentiates Mitochondrial Ca2+ Uptake Capacity

Anne N. Murphy, Robert S. Balaban*, and Gary Fiskum
Department of Biochemistry and Molecular Biology
George Washington University Medical Center
***Lab. of Cardiac Energetics, NHLBI, NIH**

Overexpression of the anti-apoptotic protein Bcl-2 inhibits the delayed death of GT1-7 hypothalamic tumor cells following transient exposure to chemical hypoxia (cyanide) and aglycemia. In addition, Bcl-2 prevents mitochondrial injury that is evident early in this death pathway (Myers, K.M., Fiskum, G., Liu, Y., Simmens, S.J., Bredesen, D.E., and Murphy, A.N., J. Neurochem. 65:2432-2440, 1995). The antioxidant N-acetylcysteine at 1 mM provides similar, if not enhanced, protection against mitochondrial respiratory inhibition and delayed cell death providing evidence that this early mitochondrial injury is oxidative in nature. Other experiments designed to assess the mechanism of action of Bcl-2 in prevention of ischemic damage have revealed a significant potentiation by Bcl-2 of the maximal mitochondrial Ca^{2+} uptake capacity, which is most dramatic when NAD^+-linked substrates are provided for oxidation (Murphy, A.N., Bredesen, D.E., Cortopassi, G., Wang, E., and Fiskum, G., Proc. Natl. Acad. Sci., in press, 1996). Bcl-2 overexpression is associated with higher levels of membrane potential (as measured by TPP+ sequestration) and an enhanced ability to maintain membrane potential and re-reduce pyridine nucleotides following Ca^{2+}-induced oxidation. Cyclosporin A (20 micromolar), an inhibitor of the mitochondrial membrane permeability transition, potentiates the Ca^{2+} uptake capacity of mitochondria in both control cells and Bcl-2 overexpressors, and decreases the difference in maximal sequestration between the two mitochondrial types. These data support a specific protective effect of Bcl-2 on retention of normal mitochondrial function in response to high Ca^{2+} loads or oxidative stress which may provide a mechanistic rationale for the use of agents designed to protect mitochondrial function in the treatment of ischemic injury.

PREVENTION OF pH-DEPENDENT REPERFUSION INJURY

Dr. John J. Lemasters
Department of Cell Biology and Anatomy
University of North Carolina School of Medicine

My research interests concern cellular mechanisms underlying hypoxic and toxic injury to liver and heart cells and organs stored for transplantation surgery. In particular, my laboratory is applying new techniques of laser scanning confocal microscopy to characterize ion homeostasis, mitochondrial function, protease and phospholipase activation, stress protein expression, and lysosomal breakdown during the pathogenesis of lethal cell injury. A recent and exciting new finding is the demonstration of a "pH paradox" in ischemia/reperfusion injury. The pH paradox refers to the paradoxical worsening of cell injury when pH is returned from acidotic to normal during reperfusion. This change of pH rather than reoxygenation precipitates lethal cell injury after reperfusion. The pH paradox is likely mediated by activation of pH-dependent degradative enzymes. Significantly, lethal cell injury caused by the pH paradox can be prevented by inhibition of Na^+/H^+ exchange in the plasma membrane.

We are also characterizing the role of the mitochondrial permeability transition in toxic and hypoxic injury. Increases of mitochondrial free Ca^{2+} and oxidation of mitochondrial pyridine nucleotides and glutathione promote the mitochondrial permeability transition that, in turn, leads to mitochondrial depolarization and uncoupling of oxidative phosphorylation. Recently, we showed the occurrence of the mitochondrial permeability transition in models of oxidative stress and reperfusion injury. Furthermore, inhibitors of the permeability transition, like cyclosporin A and trifluoperazine, reduce lethal cellular injury during oxidative stress and after reperfusion of ischemic cells. These findings offer new strategies to rescue cells and tissues from irreversible toxic and ischemic injury.

We are also studying reperfusion injury to livers stored for transplantation surgery. Following periods of storage associated with graft failure after transplantation, we showed that reperfusion causes sinusoidal endothelial cells to lose viability and Kupffer cells (hepatic macrophages) to become activated. Based on these findings, we developed a new solution, Carolina rinse solution, whose use during reperfusion reduces lethal endothelial injury greatly and improves graft survival dramatically. This solution is now in clinical trials. We are further pursuing mechanisms of Kupffer cell activation caused by ischemia/reperfusion, endotoxin and traumatic stress, particularly the roles played by calcium and potassium channels, endocytosis, adenosine receptors and nF/κB activation.

Expression of Heat Shock Proteins in the Gut after Hypoxia and Exogenous Stress

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Department of Obstetrics and Gynecology and Genetics Development
Columbia University, New York

The long range goal of our experiments is to characterize the role of the hsp genes in the cellular response to hypoxic cell damage in the gut and to determine the function of the hsp in the cellular adaptation to hypoxic conditions and the development of tolerance to subsequent hypoxic assaults. We have examined the spatio-temporal pattern of expression of members of the hsp cellular stress gene family at the protein level in the gut of adult mice subjected to experimental hypoxia and heat shock. Immunocytochemical detection in histological sections showed induction of the Hsp 70 inducible family member in the mouse stomach after 2, 8 and 16 hours of hypoxia. The expression was observed in Chief cells, which secrete pepsinogen, and in Parietal cells, which secrete hydrochloric acid. Expression of Hsp32 (heat shock protein encoding Heme Oxygenase-1) was detected in the stomach at 8 hours after heat shock, in Parietal cells and in surface mucous cells of gastric pits. Expression of Hsp25 was induced by hypoxia in all regions of the mouse gut examined, including stomach, small and large intestine. In the stomach, strong induction was detected in squamous epithelium. In small intestine, the expression was restricted to Goblet cells, which secrete mucinogen and in lamina propria of villi. In lamina propria, the expression was localized to smooth muscle cells and lymphocytes. In large intestine, the expression of Hsp25 was detected in Goblet cells and Paneth cells, which secrete lysozyme. Expression of Hsp25 was also induced in circumferential and longitudinal layers of the muscularis mucosae in the stomach, small and large intestine. The results indicate that hsps are expressed in different cellular populations and in different patters after hypoxia and heat shock. Moreover, cells with high secretory functions appeared to be particularly sensitive to oxygen depletion. These findings indicate that hsps can serve as markers of hypoxic injury in different cellular populations of the gut. We are currently generating transgenic mice to assess how gain and loss of function mutations of several hsp genes in the mouse will affect the mechanisms of cell adaptation and recovery after experimentally induced hypoxia.

Expression of Stress Glycoproteins and Heat Shock Proteins in Renal Proximal Tubule Cells after Transient Hypoxia

Kurt J. Henle, Ph.D.
University of Arkansas for Medical Sciences
and John L. McClellan Mem. Veterans Hospital
Little Rock, Arkansas

The objective of this study is to characterize the regulation of J6/GP50 gene expression and glycosylation in response to renal ischemia and its relationship to expression of the major heat shock protein, HSP70 during hypoxic stress. A secondary objective is to induce the cellular stress response and upregulate J6/GP50 and other stress proteins for the development of ellular resistance to hypoxic damage. Initial efforts included the assessment of a potential therapeutic value of retinoic acid and a thiozolidine prodrug of cysteine, RibCys. Our data show that in vivo, J6 and its glycosylated form GP50 appear at 1-5 days following a renal ischemic episode of 45 min. Modeling of this response in vitro in a short-term primary culture model shows that standard culture conditions of freshly isolated proximal tubule cells is not adequate for simulating the in vivo features of the cellular stress response. A comparison of the stress response in freshly isolated tubules, in tubules grown under oxygenated conditions on plastic, or under standard culture conditions indicate the progressive capacity of the cells to respond to stress as they adapt to non-physiological conditions. This is reflected both in the accumulation of -classical- heat shock proteins and in the accumulation of stress glycoproteins. Mechanistic studies of these two components of the stress response therefore are expected to be sensitive to the details of the cell culture model. In conclusion, the ischemic/hypoxic stress response and the heat stress response involve both HSPs and stress glycoproteins. Their role in protecting the kidney against ischemic injury, and their interaction with each other remain to be defined.

Role of Endonuclease in Hypoxic Injury

Sudhir V. Shah, M.D.
University of Arkansas
Little Rock, Arkansas

I have been interested in the mechanisms of renal injury and most specifically the role of reactive oxygen metabolites in models of acute renal failure including ischemic acute renal failure. It has been generally accepted that the DNA damage induced by oxidant stress was due to the site specific generation of hydroxyl radical or other oxidant species on DNA.

Based on the ability of oxidants to increase intracellular calcium prior to any evidence of cell injury (Am J Physiol 263:F214-F221, 1992), we reasoned that endonuclease activation may play a role in the DNA damage. We demonstrated that exposing renal tubular epithelial cells (LLC-PK1) to hydrogen peroxide led to DNA fragmentation and DNA damage that was prevented by endonuclease inhibitors (J Clin Invest 90:2593-2597, 1992). This led us to consider the possibilities that endonuclease activation, classically considered to be associated with apoptosis, may be important other forms of cell death traditionally felt to result in a necrotic form of cell death.

We examined the role of endonuclease activation, considered a characteristic feature of apoptosis, in hypoxia/reoxygenation injury to rat renal proximal tubules. We demonstrated that subjecting rat renal proximal tubules to hypoxia/reoxygenation resulted in DNA strand breaks and DNA fragmentation which precedes cell death. Hypoxia/reoxygenation resulted in an increase in DNA-degrading activity with an apparent molecular mass of 15 kDa on a substrate gel. Despite unequivocal evidence of endonuclease activation, the morphologic features of apoptosis were not observed. Taken together our data provide strong evidence for a role of endonuclease activation as an early event which is entirely responsible for the DNA damage and partially responsible for the cell death that occurs during hypoxia/reoxygenation injury (Proc Natl Acad Sci USA, 92:7202-7206, 1995).

Our current studies are targeted towards understanding the mechanisms involved in the endonuclease activation. Cultured cells are very valuable for assessing mechanistic issues and have been extensively utilized to study hypoxic injury. Hypoxia resulted in an increased DNA-degrading activity with a molecular mass of approximately 15 kDa, and led to DNA strand breaks and DNA fragmentation that preceded cell death. Endonuclease inhibitors prevented DNA strand breaks, fragmentation and cell death (Kidney Intl, 49:355-361, 1996). These studies indicate the suitability of hypoxic injury to LLC-PK1 cells as model system to study the mechanisms involved in hypoxic injury.

In our recent studies, we have examined the role of reactive oxygen metabolites in hypoxic injury to LLC-PK1 cells. LLC-PK1 cells subjected to hypoxia resulted in enhanced generation of intracellular reactive oxygen species. Scavengers of reactive oxygen metabolites and metal chelators provided significant protection against hypoxia-induced DNA

Calpain Proteases as Effector Mechanisms in Anoxic Hepatocyte Injury

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Mayo Clinic
Rochester, Minnesota

Calpain proteases contribute to hepatocyte necrosis during anoxia. Our aims were to ascertain the mechanisms causing calpain activation during anoxia and to determine the mechanism by which they may precipitate cell necrosis. In rat hepatocytes, a 2-fold increase in calpain activity occurred despite the lack of an increase in cytosolic free calcium. The increase in calpain activity was not associated with an increase in calpain messenger RNA or a decrease in calpastatin messenger RNA expression. Because phospholipid degradation products generated by phospholipases can activate calpains at physiologic calcium concentrations, we determined the effect of phospholipase inhibitors and activators on calpain activity. The phospholipase inhibitor fluphenazine, decreased calpain activation and improved cell survival. Melletin, a phospholipase activator, increased calpain activity and potential cell killing. These data suggest a novel cascade for degradative hydrolase activity during hepatocyte necrosis by anoxia with phospholipase-mediated activation of calpains. The mitochondrial membrane permeability transition (MMPT) has been proposed as a mechanism of cell necrosis. Therefore, we next determined whether calpain-like protease activity may induce MMPT. A calpain protease inhibitor inhibited both calpain-like protease activity and induction of the mitochondrial membrane permeability transition by calcium. This effect of the inhibitor was specific. The protease inhibitor also delayed the onset of mitochondrial depolarization and cell necrosis during treatment of rat hepatocytes with tertbutylhydroperoxide. These data suggest a unified hypothesis linking calpain-like protease activity to the mitochondrial membrane permeability transition in cell necrosis. We propose that in this degradative hydrolase cascade with phospholipase mediating activation of calpains that mitochondria are the target of calpain activity which then leads to cell necrosis.

The Role of Bcl-2 Homologues in Hypoxia and Growth Factor Starvation Induced Apoptosis

Robert K. Winn, Aly Karsan, Carol J. Cornejo, and John M. Harlan
University of Washington, Seattle

Endothelial cells are uniquely positioned to encounter a variety of substances that might cause injury to these cells, including humoral factors, mechanical factors and inflammatory molecules. However, these cells have a very long lifetime with turnover rates being months to years. Thus, cell death must be a rare event for endothelial cells suggesting that these cells are protected from programmed cell death (apoptosis). We have examined cell death resulting from apoptosis induced by growth factor starvation and hypoxia. Human umbilical vein endothelial cells (HUVEC) in separate experiments were subjected to a hypoxic environment or growth factor withdrawal to induce apoptosis. We have shown that hypoxia requires approximately 48 hours to induce apoptosis leading to the conclusion that these cells are resistant to hypoxia relative to other cells types. Northern blots of RNA from hypoxic cells at 24 and 36 hours had increased expression of the Bcl-2 homologue A1 and the cytoprotective molecule A20. Expression of mRNA at 48 hours for these molecules has decreased back toward baseline. These data are consistent with A1 and/or A20 being cytoprotective soon after initiating hypoxia. Addition of basic fibroblast growth factor (bFGF) to starvation medium prevents apoptosis and cell death beginning as early as 3 hours after addition of bFGF. Western blot analysis shows an increased expression of the cytoprotective molecule Bcl-2 at 6-9 hours. There was no increased expression of other known members of the Bcl-2 family. These data are consistent with Bcl-2 providing late protection in HUVEC but do not explain the early protection.

Mechanisms of Death in Cerebral Endothelial Cells in Response to Hypoxia, Hyperoxia, or Inflammatory Signals

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Cerebral endothelial cells (CECs) are the interface between blood/blood cells and the brain. CECs die by necrosis or apoptosis under different experimental conditions. CECs are the major site of disturbance in a number of pathological states including hypoxia, hyperoxia, or inflammatory/infectious disorders which are frequently encountered by Navy personnel. We explored mechanisms of CEC death in response to hypoxic or hyperoxic condition or inflammatory signals. Apoptosis can be induced in CECs by exposure to hyperoxia or following sequential treatments with tumor necrosis factor alpha (TNFa) and cycloheximide (CHX). CEC death triggered by hyperoxia or TNFa/CHX shared a number of common features of oxidative stress: DNA fragmentation and laddering, positive TUNEL stain, and an increase in NF-kB binding activity. DNA fragmentation in CECs induced by hyperoxia can be inhibited by N-acetyl-cysteine, melatonin, or a novel antioxidant, carboxy-buckminsterfullerene. CEC death induced by hypoxia is primarily a necrotic process which is characterized by early membrane breakdown and release of LDH in contrast to apoptosis triggered by hyperoxia and TNFa/CHX which is associated with early DNA fragmentation in the absence of LDH release. Understanding the differences in mechanism of CEC death under various pathophysiological conditions may aid in the future development of specific therapeutic strategies directed at particular disease states which are more commonly encountered by Navy personnel. (Supported by an Office of Naval Research Grant: N00014-95-1-582-01)

ORALLY ADMINISTERED IL-6 AS PROHPYLAXIS FOR SEPSIS FOLLOWING HEMORRHAGIC SHOCK

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Orally administered IL-6 has been shown to reduce bacterial translocation from the intestines of hemorrhaged mice and rats. The mechanism(s) for such beneficial effects have not been elucidated. We investigated the ability of oral IL-6 to affect microcirculation in the ileum following hemorrhage. Doppler flow measurements, as well as E.M. studies using iv administered HRP, showed that blood circulation was markedly reduced following hemorrhage with resuscitation. Following IL-6 administration, however, the intestinal microcirculation was again patent as demonstrated by increased HRP permeability and Doppler flow. Intralumenal HRP was shown to pass between intestinal epithelial cells of hemorrhaged mice, but not in hemorrhaged mice fed IL-6 or normal mice. Since the IL-6 effect on intestinal microcirculation occurs within 3-5 minutes, as measured by Doppler flow, it is unlikely that enzymatic digestion of IL-6, which reaches maximum in 30 minutes *in vitro*, can completely abrogate the effect. We propose that a mechanism of action of IL-6 in intestinal ischemia is to relax the intestinal microvasculature, thereby allowing restored oxygenation of tissues.

TRAUMA AND HEMORRHAGE ALTER *IN VIVO* CELL PROLIFERATION, GENE EXPRESSION, AND APOPTOSIS

Dr. Thor B. Nielsen
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The form of ischemia of most direct military significance is hemorrhage. Hemorrhage is frequently associated with trauma and complicates the course of recovery and treatment. Work in other laboratories has defined the major physiological consequences of hemorrhage and important work has been done on cellular function during trauma and hemorrhage, including regulation of vascular tone and activation of cell function. Resuscitation with acellular oxygen carrier solutions offers the potential advantage of improved oxygen delivery compared to crystalloid solutions, but the cellular consequences of improved resuscitation have not been fully evaluated. The status of cell death, cell proliferation, stress protein response, and early gene expression in a living tissue or organ is pivotal in understanding the pathophysiology of trauma. Our studies evaluated local and systemic cellular effects of trauma, hemorrhage, and resuscitation in a model of combined hemorrhage and surgical trauma. *Methods:* Trauma was modeled in rats by full-thickness shoulder to pelvis mid-line incisional wounds and hemorrhage by fixed volume depletion. The *c-fos* mRNA was determined by non-radioactive *in situ* hybridization with a biotinylated rat *c-fos* oligonucleotide probe. HSP 70 was investigated by immunohistochemistry using a monoclonal antibody against HSP 72/73. Cellular proliferative responses were evaluated by labeling *in vivo* with 5-bromo-2'-deoxyuridine. Apoptosis was characterized immunohistochemically by TdT-mediated dUTP nick end labeling (TUNEL). Diaspirin crosslinked hemoglobin (DCLHb™) or shed blood were compared for resuscitation. *Results:* The *c-fos* mRNA was detected in the epidermis soon after trauma and peaked at 6 h post-injury. DCLHb resuscitation modified the *c-fos* mRNA expression in epidermal keratinocytes. HSP 70 was rapidly expressed in epidermis and hemorrhage enhanced expression. Trauma inhibited keratinocyte and hepatocyte proliferation soon after the trauma, and stimulated subsequent proliferation of keratinocytes and liver non-parenchymal cells. DCLHb stimulated wound keratinocyte proliferation and attenuated the inhibition of hepatocyte proliferation. The skin cells most active in both proliferation and apoptosis appeared to be keratinocytes. Normal rat skin had low rates of proliferation and apoptosis. The ratio of proliferating to apoptotic cells (P/A), an indication of deviation from homeostasis, was strongly decreased in response to hemorrhage and closer to normal after administration of DCLHb, suggesting improved cell vitality. *Conclusion:* Trauma alone, or in combination with hemorrhage, modulated cell proliferation both in the wound and in the remote organs of intestine and liver. DCLHb enhanced wound healing and cell proliferation as well as, or better than, freshly-drawn blood, and may be beneficial for trauma care. The co-expression of *c-fos* gene and HSP 70 can be used as an indicator of pathophysiological response to hemorrhage and acute wound. The *c-fos* gene and HSP 70 expression in epidermis may be a sign of traumatic severity whereas *c-fos* induction and inducible protein HSP 70 in sebaceous glands may reflect the strength for protection. If so, DCLHb seemed

to be most effective for resuscitation in this study. These data suggest that resuscitation with blood or DCLHb is important not only for the survival of the organism, but also for skin remodeling and keratinocyte viability.

DEVELOPMENT OF CLINICALLY-RELEVANT MODELS OF TRAUMATIC SHOCK

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University of Tennessee

Despite overwhelming evidence favoring various anti-sepsis strategies in numerous experimental animal models, virtually all the large scale, multi-center trials have yielded disappointing results. Costs have exceeded tens of millions of dollars. The profound disparity between the experimental and clinical results can be attributed to one of two simple explanations: either animals react differently than humans to the same stimulus, or the experimental models do not accurately reflect the real life situation.

Consider the following: First, otherwise healthy humans do not spontaneously develop sepsis, but a typical experiment uses healthy, drug-free animals on fixed, standard diets exposed to extraordinary septic challenges in the absence of even the most basic standards of care (i.e., supplemental oxygen, fluid, ventilatory support). The pathophysiologic responses in such extreme conditions are rarely seen in real life, so it follows that therapies that are effective in those models might not necessarily apply to humans. Second, precise timing of the therapeutic intervention is critical for influencing outcome. The same treatment applied early can have opposite actions if applied late. Unlike the models, the exact time course may be difficult or impossible to track in most septic patients, so it follows that those therapies will have variable results. Third, most septic patients, unlike the experimental animals, will eventually recover, even though mortality is often the main, albeit crude, endpoint in both and clinical and experimental trials.

To address some of these problems, we have narrowed our research focus to the causes and treatment(s) of sepsis following trauma. The trauma patient, like the experimental animal, is usually healthy at the time of injury. In our models and in trauma patients, the priming insult and the therapeutic intervention can be precisely timed. Major advantages of the model are that multiple invasive variables can be monitored and the magnitude and location of the injury can be controlled. After traumatic shock (hemorrhage combined with various tissue injuries), pigs are resuscitated with protocols similar to those in most urban trauma centers. Not surprisingly, the results are variable, but remarkably similar to those in patients. We have demonstrated trauma-induced changes in pulmonary function and the efficacy of therapeutic interventions that replete high energy phosphates and which target leukocyte-mediated reperfusion injury. We have evaluated effects of alcohol or cocaine because a large fraction of trauma patients are intoxicated. We have shown the relative risk of transfusion for increasing post trauma sepsis. These results provide a firm foundation for well-designed future clinical trials of novel therapeutic strategies. In addition, these data provide insight for the development of new models that more faithfully mimic civilian or military trauma and the complications of post trauma sepsis.

PANEL DISCUSSION

Dr. Thor B. Nielsen; Naval Medical Research Institute

We've heard rather extensive discussions of cell biology and we've heard some work on animal models of trauma and resuscitation. I'd like to take two minutes and introduce a different topic, but one in which I know many of you have an intense interest. In this slide are the national costs of several major categories of disease (Figure 2). They are shown in red (cross-hatched bars, right axis) in billions of dollars, and the categories of diseases are cardiovascular diseases (labeled heart); the traumatic diseases, injuries, and accidents (labeled trauma); arthritis and musculo-skeletal diseases; diabetes; cancer; and AIDS. These data are from a study by J. Charnow from this year[1] and from work by Jaffin, et al. from a couple years ago[2]. These are broad categories of disease. These are *estimates* of the costs in the United States for treating the population that is afflicted with heart disease, trauma, and these other diseases, in billions of dollars. On the same graph (left axis, solid bars) is plotted the National Institutes of Health (NIH) budget for fiscal year 95 which is applied to research in these same broad disease categories: heart disease, trauma (including burns, general trauma, and trauma to head, CNS, and spine), arthritis, diabetes, cancer, and AIDS[3]. The NIH spends, by far, the most of any government agency on national health research. You know that the Department of Defense (DOD) also has a budget for biomedical research. It is small by comparison, but it is of interest to know that most of those funds are also spent on cancer and infectious diseases, so if you add those figures in, the overall distribution does not change very much. In times of war, casualties are likely to have mostly problems of trauma and infectious diseases of various kinds. You can see from this graph is that there does not seem to be a very close relationship between the costs of a disease and the amount expended on research to alleviate it. I hope that the panel can keep this kind of information in mind as they consider research needs for the Navy in the related fields of hypoxia, ischemia, and trauma.

The panel members are: from my left, LCDR Peter Rhee who is a *bona fide* trauma surgeon; MAJ Verma from the Uniformed Services University of the Health Sciences (USUHS), a neurologist; you've already met Dr. Jones, further introduction would be pointless; Dr. Oliver will be chairing the session, she is sitting in the middle and is easy to distinguish because she is wearing red; CDR Bennett is also from USUHS, and has been very much involved in the deployment of troops; Dr. Lemasters, you've met; and Dr. Proctor is seated on the end. So I'll turn it over to you, Madam Chairman.

Dr. Constance Oliver, Office of Naval Research

What I'm asking the panel to do, and what I'd like to accomplish in the next hour, is to have CDR Bennett and LCDR Rhee 1) give us their perspective from the Navy point of

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[1] J. Charnow. Infectious Disease News 9:1, 1996.

[2] J.H. Jaffin, H.R. Champion, B.R. Boulanger. Economic Considerations. Crit Care Clin 9:765-774, 1993.

[3] D. Ralbovsky (NIH) and Norm Oliver (NIH), personal communication.

view, and from a trauma surgeon's point of view, of some of the realities of military research; 2) put the basic research that most of you are engaged in into a broader perspective; and 3) give us some of their impressions of where there are holes and gaps, that research could be beneficial. I've also asked MAJ Verma to do the same thing from the perspective of the Army. Following that, I 'd like to turn our attention to the meeting itself. I've asked the remaining members of the panel to discuss where there may be research opportunities within the overall program that was presented today and yesterday. Are there areas that should be explored further? Are there topics that weren't mentioned at all that people think are important? We have had some discussion among ourselves about model systems. How reproducible and how translatable are model systems from one investigator and one laboratory to the other? With that introduction, I'll turn things over to CDR Bennett and ask him give you a broader perspective of the problems encountered in military medicine.

CDR Brad Bennett, Uniformed Services University of the Health Sciences

Thank you, Dr. Oliver. I'd like to thank Dr. Nielsen for initially making contact with me as well as Dr. Oliver to elucidate what are the expectations of the panel. I'd also like to thank Dr. Jones, Dr. Proctor, and Dr. Lemasters for chairing the sessions and making their presentations, and particularly to you, as researchers in the academic community, for showing the interest to draft a proposal, submit it to the Office of Naval Research, subject yourself to critique from a peer review, and being selected to make a difference for the sons and daughters of Americans: our sailors, our marines, our soldiers, and airmen. I'm in the Department of Military Emergency Medicine at USUHS in Bethesda, and our focus there is certainly to train quality medical students. However, there is a large thrust in basic research and providing the Masters degree in Public Health and graduate degrees in other departments. But my concern is focusing in on the global thrust of why we're doing this research. Dr. Proctor also presented some slides earlier today on some of the Viet Nam War data. Where are we going in the sense of war fighting strategy? Therefore, how should our health service support component of each service then support that line command, those assigned to the ships, those ground pounders or ground forces, the Marines, the Army, and also the Air Force. Our war fighting strategy is changing rapidly as we speak, and I've been fortunate in the last two months to have been in a couple high level meetings associated with that war fighting strategy. Then how are the medical departments of each service going to support the war fighting? I think you need to understand that a lot of the models, the casualty prediction models that have been used in the Desert Storm War, are really data of the past: data of Viet Nam, data of Korea, of World War II, and World War I. The Department Of Defense and all of the commanders and chiefs that are in charge of all the unified commands, like General Schwartzkopf, who you may remember very well I'm sure from Desert Storm, depend a lot on this input to get the civilian laboratories, as well as the Department Of Defense laboratories, to develop and come up with casualty prediction models based again on data in the past. When was the last time we fought a war the same way previously? The answer is were not. War is changing. War of attrition will not be the way we fight wars in the year 2000. So we'll be fighting wars very quickly, very rapidly, with overwhelming forces. Even to say the types of injuries that you see, and 98% of the injuries that occurred in Viet Nam,

are from the operational ground forces. So the thrust of research certainly should stay within that focus for the ground forces. When we talk about Navy, for some of you who aren't familiar with the military institution, we're talking about the Navy Department and that includes US Marine Corps as well as the sailors that are at sea primarily. And we have seen some data earlier today, as well as earlier in the week, about the magnitude of injuries that occur. From a retrospective analysis of those data, at war time, casualties of sailors at sea have almost 57% mortality from catastrophic accidents [as well as 50% of these are injuries]. We talked about the Stark incident earlier today and that the Exocet missile that was launched at the USS Stark from the Iraqis killed 37 people outright and injured an equivalent number. So the profile and the types of injuries we see at sea are different than we see for the ground forces. I think that one of the slides elucidated the types of injuries; blast injury, crush and traumatic injuries, burns and inhalation injuries. There are all types of other injuries that go on on a daily basis that are pretty much managed by prevention programs. Our concern is really not prevention programs. The focus of this orientation the last two days, rightly so, is casualties. There is a tremendous emphasis in military medicine to focus in on what effect the majority of the ground pounders, the Marines and Army soldiers, have to do with non-battle disease injury. That means every type of illness or injury that occurs not in relationship to mortality and morbidity associated with fighting the war. There is a tremendous amount of emphasis on infectious disease control, malaria control, and providing prophylactic treatment, and that's a whole other thrust that takes a lot of energy and a lot of research that occurs in your institutions as well as the Department of Defense laboratories. But for those injuries that occur from battle injury itself, there is a tremendous amount of focus that should be maintained on the hemorrhagic model and a tremendous focus that should be maintained on septic models. I'm not going to address the types of models you can use, but I do want to state for those who are just getting into this type of research, what we call the 6.1 level of funding, of the basic research are not be discouraged. Because in an analogy of a book salesman going door to door, you have to go through so many "no"s to get to the "yes", meaning the guy who buys the book. From a basic research approach, we have to maintain an appropriate level of funding and focus on basic research with ultimately high risk high dollar obligations with potentially a high risk payoff down the road. If you don't see a transition in the type of research you're doing, whether it's at the cellular level or the sustainment of shock type model and *in vivo* and *in vitro*, it really doesn't matter to me. Ultimately, we need to have a transition of the work that goes from the basic research ultimately to a product for the end user, that being the medic or corpsman or the 2nd or 3rd or 4th echelons of care. That meaning someone who does life sustaining intervention, maybe an emergency room physician, a doctor or a physician assistant who gets Med-Evaced, as we call it, evacuated to a mobile hospital that's set up in the theater and then ultimately maybe to a hospital ship that basically is a floating hospital, that'll do every type of intervention necessary to sustain a life. But the hospital ship will not do the types of surgery that will be of a reconstructive type. That'll be back in CONUS (Continental United States), Walter Reed and Bethesda Naval Hospital are examples of where reconstructive type surgery will go on 30 and 60 days, and it could be even longer, after injury. So the focus, I think, really still needs to be at the forward edge of the battle area: sustainment of life, maintaining critical life saving intervention. So hemorrhagic models certainly need to be there. Emphasis on trauma

soft tissue injury models needs to be maintained in the basic research. Bellamy, a physician that is due to retire if he hasn't retired already, is a Colonel in the army and he's in the area here. COL Bellamy is an adjunct professor in my department as well as in surgery with Dr. Rhee. He's established and he's a tremendous asset to have in the area and he'll continue to be a tremendous asset. But 50% of the US injuries in the Viet Nam War were soft tissue injuries with minimal to moderate severity that were not life threatening. Also there was a tremendous amount of exsanguination, people that died because of tremendous hemorrhaging on the battlefield. Fifty to sixty percent of the injuries which occurred during the Viet Nam conflict were to the extremities; traumatic injury to the extremity caused by missile fragmentation. The majority of the injuries, those who were wounded in battle and those who got to a treatment facility, really occured from missile fragmentations. Now we mostly think of small arms fire, that being a semi-automatic, an automatic rifle, or a nine millimeter caliber high velocity pistol, as being the cause of injuries to most of our ground pounders in Viet Nam. The majority of those who die, die from small arms fire. Yet a large percentage of injuries occur from fragmentation, and we think of a single type injury typically in these individuals but in reality they are multiple type injuries. They have multiple fragmentation so they may have multiple sites that they are bleeding from. The corpsman that works for the Marines and the medic that works for the Army are trained to deal with exsanguination. They know from early onset of their basic medical training that they need to deal with the hemorrhagic side of that casualty. So they may go up to that battle casualty in the line of fire and deal with that with a tourniquet. So a tourniquet model of research in dealing with hypoxia and anoxia in extremities certainly should continue to be a research focus. So this clamping, if you will, of a major vessel, then a reperfusion once he gets to a treatment facility, is going to occur. Perfusion injury research, because of that scenario, needs to continue and I challenge you to continue those lines of investigation. There is another area I just want to briefly touch on because we've talked about hypoxia induced mechanically, in this case say a tourniquet, or chemically. You've given us some sophisticated models of hypoxia in the last couple days and I applaud you for using well balanced and reproducible models. You may not see the application to the clinical relevant model at this point, but that's okay. The transition that's done in research is, as I call it, really going to be the responsibility of Dr. Oliver and her colleagues at the Office of Naval Research: to look to the next step of funding, ultimately coming up with a final product for the end user. But something that wasn't touched on, and maybe rightly so, are concerns of weapons of mass destruction. Those weapons of mass destruction are radiation syndromes from nuclear weapons, biological infections from anthrax, botulinin toxin, plague, so on and so forth, and many others, as well as the chemical injuries that are induced. Sarin, Soman, VX and Tabun are your classic nerve agents that are used by 3rd world countries, and agents all induce rapid death. The LD50 (the lethal dose of 50% of the population) for nerve agents is 10 micrograms, that's just a dot of fluid on a penny: very, very small. These agents will cause rapid cell death in the bone marrow, and so we have already talked about cytokines and cytokine therapy. Cytokine therapy for a nerve agent patient as well as the radiation syndrome patient (lowest level being hematopoietic syndrome) in which cytokine therapy stimulates stem cells is a very important type of research. So I challenge you to not only look at a univarient model in your research, but a multivariate model just as the Armed Forces Radiobiology Research Institute right here in

Bethesda is now looking at the synergistic effect of acute radiation syndromes all the way from the low level hematopoietic effects to the CNS model, which is around 900 centigray of radiation and induces death very rapidly through a cascade of effects. But they're looking at the synergistic effects of a biological weapon, as well as radiation syndrome, both suppressing immunological functions. If a traumatic injury occurred first and then the radiation or, conversely, the radiation first and then the traumatic injury, the response and the mortality of those types of injuries are completely the opposite. I also challenge you to take a look into collaborative efforts looking at injuries as you showed in some of your cellular models. If you are exposed to a pulmonary toxic agent, e.g., phosgene, and blood toxins such as cyanide, again we're inducing hypoxia by weapons of mass destruction. When chemical agents are combined with hemorrhage and trauma, are the physiological cascade of events the same or are they different? So we need to broaden our perspective to look at synergistic injuries with weapons of mass destruction. I think with that note I'm going to stop, Dr. Oliver, and let someone else take over.

Dr. Constance Oliver

Thank you very much, I'm going to change tactics a little bit. I'd like to have Dr. Rhee give us his impression of opportunities and problems from the perspective of a trauma surgeon who would be ultimately, medically, the end user of any products that we hope to develop from this program.

LCDR Peter Rhee, Uniformed Services University of the Health Sciences

Thank you, Dr. Oliver. First I'd like to say, Dr. Oliver and Dr. Nielsen, that this is a very high quality meeting and I've really enjoyed the presentations. I'd also like to applaud all the people who have presented their work. What I'd like to talk about today is basically who I represent and what I, as a commissioned officer, would like to see come out of these types of meetings and research. Keeping in mind that I also do research myself so I have my own inherent biases, I'd like to say that I represent not just the Navy but coming from the Department of Surgery at USUHS, but I think of myself as more tri-service. I think that just like Dr. Oliver stated, even though this is Office of Naval Research and the Navy research funds, they're the only ones that fund combat casualty care in the military. So I first want to say that when I think about who my patients are going to be, it's not going to be just the Navy personnel on the ship, but I think most of the time its going to be the Marines who are getting shot and blown up. It is also going to be the Army and Air Force people as we get more and more tri-service, along with the prisoners of war. I know that when I'm out there in the fields and doing trauma surgery those are the types of populations I can expect. The type of injuries that I can expect are people who are basically shot or blown up or burned and I don't have to worry about too much of the other things. And since we're doing research in combat casualty care, that's basically just trauma research and trauma care. So when I think about that I like to always go back on the data that is available. You know, when you think about four or five different scenarios in the future as to what the military scenarios are going to be, that's really difficult to say. But since I do fundamentally just think about people who are shot

and blown up, when it comes to it, the data that is available and is still relevant to a certain degree, is from the Viet Nam data that we have from Dr. Bellamy. This data shows you that one of the major contributors of death and one of the most important things that we think about is hemorrhagic shock. I think about everything back to that area of research and say "how does this apply to hemorrhagic shock to protect us". So I always think about hemorrhagic shock as the fundamental problem that I need to see and care about. So I always think about how is this going to affect my therapy, or is this going to change my practice? I mean, from the Korean War we learned how to give blood to save lives and from the Viet Nam war we learned how to give crystalloids and prevent renal failure and at the same time delaying death. So if you look at where the majority of the deaths on the field occurred, they did occur on the field, and they occurred from hemorrhagic shock. So when I hear about things such as hypoxia, I ask myself "do my patients get hypoxic and how important is the mechanism of hypoxia in my patients". So I think that one of the most important things when we study hypoxia, for example, is to be always able to keep an eye on the bigger picture itself and show me how this is relevant to me. If I hear that some things are just reproducible in very unrealistic environments then I don't really know if it would be applicable for me as a trauma surgeon who also does critical care on patients afterwards. I just want to say that I think it's really important to keep the clinical picture in mind. Also, knowing that research has begun in all ranges, meaning down at the cellular level and all the way up to the clinical level and one can't be done without the use of the other, so that has to be all married together. I think that's probably the biggest key. I'd like to say that if we can all, instead of just being segregated by ourselves, use collaborative efforts both as clinicians and also basic scientists, to keep an overall picture of where we're going with this, and ask ourselves "is this data going to be of any use or is it going to have any relevance to the future"? And that's all I really have to say.

<u>Dr. Constance Oliver</u>

The next person that I'd like to hear from is MAJ Verma, his perspective both as a researcher and as a representative of the army.

<u>MAJ Ajay Verma, Uniformed Services University of the Health Sciences</u>

I might add that I'm also a clinician so I agree with some of the things my colleague said. The brain suffers hypoxia not only in hemorrhagic injury. The head is a closed vault, so any kind of space-occupying lesion in the brain, whether its a blood clot or any kind of trauma, diminishes blood flow and results in significant hypoxia to the brain. Hypoxia is a big issue in neurology and I applaud all the work that's being done in this area. From the Army's perspective, I think the interest is in very far forward research. They are not big fans of basic research and want research that can be applied immediately to help the soldier that's down and out in the battlefield. They're interested in trying to get findings quickly from the bench to the clinic or to the field application. In that regard, I think there are certain areas of research that are more exciting than others. Some research tends to effect changes in the paradigms in the way we actually treat patients. One area that comes to mind in brain injury for example,

is the study of secondary cascades of injuries that happen following the initial injury. That gives us an opportunity to intervene, and I think that's changed the view of a lot of clinicians as far as what they can do for brain injury. There are other paradigms that we've heard about, I think, at this meeting. For example, instead of focusing on calcium overloads perhaps we should focus more specifically on the mitochondrial permeability transient. The concepts of delayed cell death and programmed cell death, I think, are very important to appreciate in terms of really getting a handle on how we should approach treatment of a patient. Research that focuses on transition between basic research and clinical applicability should also be applauded. We need to have some clinical markers that tell us which animal research models are applicable to the clinical situation. Any kind of research that can be done to link the bench top to the clinical arena should, I think, deserve a lot of focus. The other thing is that there are many targets that have been identified, as targets of intervention in preventing hypoxic cell injury. Several of these targets, I think, could be attacked with drugs that are already available clinically. There's a lot of effort sometimes spent on developing new drugs, but there are several old drugs that we're well familiar with as clinicians (we know how to use them, we know the toxicities, and so on) that could possibly be used in the same arena. I think we ought to focus a little more on that because that will help us get the product to the market much faster. Well, I think I'll stop there because a lot of the things that I wanted to say have already been said by my colleagues.

Dr. Constance Oliver

What I'd like to do now is to take any questions to myself or to the panel specifically on the military relevance of this program that any members of the audience may have.

Dr. Sudhir V. Shah, University of Arkansas

One thought, in reference to hemorrhagic shock and sepsis. I've heard reference to soft tissue injury but I've heard very little about muscle injury, *per se*. I would have thought that would be of major interest to the Armed Forces. Am I misunderstanding you or is that not a major interest?

LCDR Rhee

Well, the correct therapy for limb injury is just direct pressure. So as Dr. Bennett talks about, we're not going to have all out fights like we used to have. Rather there are going to be control type actions. So certainly the recommendations of how we treat limb injuries are different. And, for example, when we have special forces that go out, although the current therapy is not to put tourniquets on, tourniquets may become a reality in the future and be useful, especially when we think about lives being saved at the expense of a limb. So for muscle, *per se,* it's usually epitonic injury and then there's usually another secondary injury associated with it. Soft tissue injury has not caused much morbidity in the past. So I'm in agreement that's it's not much of an issue. As far as the mechanisms of sepsis or hemorrhagic shock, I'd like to take this one second to say that I think there's a very distinct difference

between exsanguination, hemorrhagic shock, anemia, hypoxia, and ischemia. So when we talk about hypoxia from total occlusion and reperfusion, for example in transplant therapy or areas such as stroke, it's different than hemorrhagic shock where we don't really have full occlusion. For me as a clinician and critical care specialist, I don't really worry about true hypoxia because its very difficult to get hypoxia at the tissue level after hemorrhagic shock or during hemorrhagic shock itself.

Dr. Constance Oliver

Any other questions? What I'd like to do at this point is take this opportunity to get an assessment, both from the panel and from the members of the audience, of where we are within the field scientifically given the caveats of where we would like to ultimately go with a clinically relevant product. Also, I would like to ask the members of the panel and the audience that, whatever their particular model system, they control the urge to say *that's* the most important thing that's being done scientifically and *that's* where all the emphasis should be placed. I'd like to try and get an objective feeling from everybody including the members of the panel in term of needs and stats and caveats. I'm going to start with the members of the panel. I think you're all coming from different perspectives, John, Dean, and Ken.

Dr. Ken Proctor, University of Tennessee

One point I have is: before we begin evaluating something, don't we have to identify the problem? You state that the battle of the future is going to be different than the battles of the past, but the models we're working on are based on the statistics of the past. Do you think there is a problem with those statistics?

CDR Bennett

Yes, let me just enlighten you a little bit more about some of the war fighting strategy changes as they're going to be fought in the year 2000 and beyond. The Marine Corps is currently working on war fighting strategy for 2010. If our line counterparts, those who are trigger-pullers (for example, aviators, submariners, tankers, etc.), if they're changing their war fighting strategy then the health service support component of each service also has to follow along in parallel. And so you may have heard in the media that the blue water Navy now has moved into the littoral, the shallow water for lack of a better term, and changing some of their service platforms to get more shallow, to bring the forces in closer. A lot of the hardware development is to support that new war fighting strategy. Analogous to that concept is what happened just recently in Iraq. Why send in the most critical assets that the Department of Defense has, that means the warm-blooded sailor, soldier, airman, our sons and daughters, so to speak? Why not use stand-off technology? Certainly that is the best way to fight a conflict. However, sometimes, as we know, we do have to put a body in the field, to go in and meet our objective. So the concept of fighting a conflict today is going to be very rapid and very mobile. So the health service support and all the other logistics support of the battle has to be able to keep up. The magnitude of injuries that we're going to see are

going to be lower certainly. The casualty prediction models that have been used over the years are based on previous war time data so they are not necessarily going to reflect the new war fighting strategy. But the types of injuries, and that's the bottom line, that we're going to see are still going to be the same types. You're still going to have a hemorrhage injury on the battle ground. You're still going to have massive trauma. You're still going to have muscle injury, but you're not going to see the muscle injury or the crushing injury on the battlefield, but on ship where the industrial type injury does occur. Again we're talking about percentages of injuries, so should muscle injury be a major thrust? I'd have to say it's probably not a major thrust, but the types of focus that you have now (sepsis, hemorrhage, and hypoxia models), will be relevant for the year 2000 and beyond.

Dr. Ken Proctor

A follow up question concerns the application of this technology to civilian use. For example, since we don't have the Cold War anymore, we're liable to have use of the military for other disasters, such as Hurricane Fran. If the military helps to care for civilian injuries, it seems that a lot of this technology might apply to the types of injuries that would be seen in the urban trauma center. I wonder if you could comment on that? These are not planted questions by the way.

CDR Bennett

The term "dual use" really came foremost in the Department of Defense under the current administration. Some of the thrust and focus of research dollars to support dual use started at that time. Yes, there are tremendous amounts of technology transfer that can occur in the Department Of Defense and be utilized in urban setting. It is relevant. I just want to point out for some of you who aren't familiar with some of the data by Bellamy and others, this Journal here is Military Medicine. It might be a particular journal that you might find to use to set up your Introduction, set up your review of the literature, that may put a military twist to your future proposals and give them the right flavor. In this particular journal you'll see articles by Bellamy, Carey, Dunnigan, and others who write on combat casualty care. This particular supplement I have right here, I brought in for Dr. Rhee based on talking to him yesterday. The military is moving very rapidly in fluid resuscitation on the battlefield for use by the forward advanced corpsman or medic. This particular thrust has to do with the special forces medic, those who support Navy seals and the Green Berets and those types of individuals. They have already set in here an algorithm[4], if you will, based on the ABCs, and fluid resuscitation being part of the ABCs, on when they conduct stages of care, e.g. care under fire, tactical field care (meaning you're out of the direct line of fire but you're still in a tactical environment), and then care under a Med-Evac or evacuation from the battlefield. There are three sets of algorithms, so this should give you some focus. In this particular thrust with the Special Forces community, a tourniquet is a primary focus for managing hemorrhagic shock.

[4] Butler FK, Hagmann J, Butler EG: Tactical Combat Casualty Care in Special Operations. Military Medicine 161, Suppl 1:3-16, 1996.

Dr. Dean Jones, Emory University

I'll just make a comment. Concerning the issue of acute anoxic injury, that is, the immediate failure of cell function, it's really one of the questions here that is of most relevance. If you can't get the cells or organs to survive long enough to get to prevent irreversible loss of funtion. One of the things that came out clearly in the talks is that there really are variables. The work like Dr. Weinberg presented and the solutions such as John Lemasters is working on suggest there are a variety of ways that one can manipulate how fast cells die. What we've looked for over the past twenty to thirty years is some miracle compound that we could add and would provide that additional time to get supportive treatment. I think it's out there. I think that we just haven't found it, and one of the problems has been in the movement of science. Science goes through fads and we've gone through a 20 year period where metabolism has been *passe*. I think that we are at a stage of knowledge now on cell death and mechanisms involved in cell death that we have some real possibilities for answers. I may be optimistic. I know that comment is self serving, but in terms of my own research, I've looked for years at the issue of chronic hypoxia and now realize that's irrelevant to the question of survival during short term anoxia. That was one of the points I tried to make. The anoxic model and the reoxygenation model are the ones that we have to deal with. We have real possibilities through basic research to come up with some more testable compounds. That may not provide us an immediate solution but it certainly is something that we have to invest in.

Dr. John Lemasters, University of North Carolina School of Medicine

I would like to address an issue related to our scientific tradition. As scientists, we are trained to look for the mechanisms underlying the particular problem at hand. Typically, we focus on one mechanism in an effort to establish how such a mechanism influences the outcome of some process, for example hypoxic injury. Similarly, drug companies are motivated in the same way. They are looking for that one drug that will cure a particular disease. Ideally, this drug acts by a single, well understood mechanism. In medical school we are taught the dangers of polypharmacy. Avoid using more than one drug so that adverse drug interactions, development of resistance to antibiotics, or simply overmedication does not develop. But for really sick patients, polypharmacy is the rule, not the exception. Patients with severe trauma are also very sick, and I think it is fundamentally wrong to expect that somehow there is going to be one drug, one mechanism, or one receptor to fill which will change profoundly the outcome of cellular injury. To rescue tissues from hypoxic and traumatic injury, we will need to go against the grain of our academic tradition. First, we have to find those things that work no matter how impractical they may seem at first. If you do not have something that works, practicality is no issue. Second, we will likely find that there are no silver bullets, no single mechanisms that explain everything. Many ideas have been expressed over the last two days that show promise to be important, even crucial in the development of irreversible cell injury. Sometimes our debate becomes the question of which idea is the best or most important. My belief is that we will find that all these ideas are important. Moreover in complicated clinical situations, we will need to apply all these ideas

to achieve a good outcome. In other words, each of the several mechanisms contributing to injury will have to be neutralized to see therapeutic success. Countering one mechanism alone may be completely ineffective, and we are prone to conclude wrongly that such a mechanism is unimportant in pathogenesis. Rather, multiple approaches and multiple drugs, i.e. polypharmacy, will be needed to treat very sick patients and their sick cells and tissues, because multiple measures are required to combat multiple pathogenetic mechanisms.

Dr. Constance Oliver

I'd like to ask a question to the panel and members of the audience. You've talked about tactical approaches or polypharmacy. Do we need different treatments for each organ?

Dr. Greg Fahy, Naval Medical Research Institute

What is the limiting organ in hypoxia and ischemia? Is it the brain? Should we be doing brain research?

Dr. Constance Oliver

Well, that probably depends on the type of injury. One thing I haven't talked about or alluded to is that the funding depends on requirements. The NIH funds brain research, so Department of Defense generally does not, except for a Congressionally-mandated plus-up of head trauma.

Dr. Sudhir V. Shah

There are a number of investigators around the country who are studying hypoxia or other areas of interest to the Navy in different organ systems such as the kidney, the brain, and the gut. Perhaps one of the roles the Armed Forces can play is to bring these investigators together and coordinate an effort centrally with the Navy. This is not the type of coordinated effort that is funded by NIH or other organizations and, therefore, may be a very special role for the Navy.

Dr. Constance Oliver

That's a point well taken. I'll say one of the purposes of this conference is to bring people together from different disciplines. It's not as broad and overarching as what you're suggesting but it's very valuable to bring people together with different perspectives in dealing with a common problem.

MAJ Verma

I agree with Dr. Shah's comments. I also wanted to add that we may learn a lot from nature's examples. There are animals that are well adapted to prolonged periods of hypoxia.

There are several groups doing work, Dr. Hallenbeck's group in particular, on animals that hibernate or estivate. We haven't heard a whole lot on the adaptive mechanisms the different organs employ in response to hypoxia, but we may learn a lot from studying animals which adapt. We heard about heat shock proteins and immediate early genes, but not much about how that relates to clinical settings. Perhaps there might be another focus on what's going on in the organs when soldier is down and out and hypoxic to adapt to that stress? Are we actually doing more harm by intervening in a certain way than we should? I also think that studies aimed toward the oxygen sensor and signal transduction mechanisms that detect changes in oxygen levels should be a main focus. Such a knowledge may allow us to modulate hypoxic responses therapeutically or prophylactically.

Dr. Constance Oliver

There is another area that several speakers alluded to, the idea of preconditioning. Certainly in a military setting, where you know you're going to be sending people into a situation where they have a high potential of suffering from injury, is preconditioning practical? For instance, could induction of heat shock protein be useful for preconditioning? Is this something that we should be considering? Is this even practical?

LCDR Rhee

My first question about preconditioning is will we give the guys some agent and heat them up before they go out and fight? In reality, the scientific question is about hypoxia and preconditioning for hypoxia. Is that a preconditioning or is that a priming? In certain circumstances that's more detrimental, but in other circumstances, if its preconditioning, it would probably be beneficial. So as a clinician, when I hear about that first initial phase, whether it be priming or preconditioning, In some instances the initial phase is protective and is called preconditioning, on the other hand the same initial phase is thought to be priming which is often detrimental. So in reality, which one is it? Priming or preconditioning? I wondered if some of the people involved with these types of projects could answer those types of questions for me?

Dr. Constance Oliver

Would anybody care to comment? This is an area that we've obviously paid no real attention to and I think that's a good point. When talking about preconditioning, it sounds wonderful in a test tube or even in a rat to produce heat shock protein, but we could ultimately be making the situation worse in a trauma injury. I think that Ken's models show what you do to a septic animal is very different from one that's been traumatized.

Dr. Kurt Henle, University of Arkansas

I just would like to point out that, of course, with a cellular stress response there are so many possible ways of inducing it non-traumatically that we really would not have to think

about preheating our soldiers. I think it would be much more realistic to think about taking some vitamins or retinoic acid, or something like this, which actually may help induce some organ-specific stress response. If you knew that you were going to be under attack, taking a pill, or something like this, would just statistically improve your chances for recovery and for survival and would be a cost effective and strategically logical approach to minimize the injury. But Dr. Lemasters is correct, in a sense, when he said we can't predict where the real breakthroughs will come. If we study the mechanisms and if we have the applications in mind, that's about as good as we can do. We really don't know where the benefits will accrue.

Dr. Constance Oliver

I appreciate your comment.

CDR Bennett

I think the notion of pretreatment is pretty exciting. It's far reaching and I challenge you not to get discouraged about approaching the concept of pretreatment. Pretreatment may be considered prophylactically, as we did with the weapons of mass destruction when we had significant intelligence on Iraqi forces and X amount of biological weapons fielded in their missiles. There was evidence that they had used them on Kurds during the Iran-Iraq war. FDA approved or not, we inoculated a significant portion of the ground forces for anthrax and botulinin. I have an interest in heat stress from a treatment standpoint, as well as a preventive measure. What did we do with Marines when they got there? No, we didn't stick them in warehouses to sit down and get ready for the war six or eight months later. They went right out into the 120 degrees dry inland heat. That's 120 degrees, very humid on the coast. They went right out and started working and got prepared from a heat acclimation standpoint. Heat acclimation is something that Marines understand. Heat acclimation actually increases the fluid volume requirements because one of the hallmarks of heat acclimation is not only reduction in core temperature and heart rate each day that you're exposed to that temperature, but also the total amount of sweat is actually increased. Fluid volume intake is actually increased in this kind of scenario. I wrote down a note when we had the heat shock protein presentation. I don't know the literature well in this particular area, but I'm questioning when the human gets heat acclimated, is there a protection from heat shock proteins associated with that as a precondition? If someone could answer that for me, I'd like to have a discussion with you. Also in weapons of mass destruction another condition or pretreatment is pyridostigmine bromide which is a pretreatment for particularly soman the nerve agent because of the aging concept. It is associated with the binding of soman to acetylcholinesterase and protects that particular enzyme to make it viable to cut down on the transmission of the acetylcholine on the postsynaptic cleft and all the associated cascade events occurred and acetylcholinesterases is essentially ineffective so there is another example. I caution you, the reason I'm talking about this is that we have a problem associated with Gulf War Syndrome. We're not authorized to use that term because there is nothing in the epidemiological data base that suggests that there ever was a syndrome. An animal model suggested that a possible problem with pretreatment or conditioning is pridostigmine bromide

as the pretreatment, Deet the agent you use on your skin to minimize insect infestation and malaria and so on, used in combination with permetherine which is another insect repellant spray for your clothing that soldiers use. The synergistic effect of all three of those potentially have a very high toxic response in the body, at least in the animal model and I think a lot of dollars are focussed on this to see if, in fact, there is any relevance to what might be the syndrome occurring in many of our soldiers and sailors.

Dr. Anne Murphy, George Washington University Medical Center

I think that the preconditioning idea is not necessarily only applicable in the combat field. I think that as a collaborator with clinicians there is certainly a constant concern. There are groups of people that we know are going into surgery when you know that certain parts of their body are going to be hypoxic for some period of time. I think the idea of preconditioning is not only applicable here, but for patients that are going to undergo certain types of surgeries. So we should keep in mind that it has more general applications.

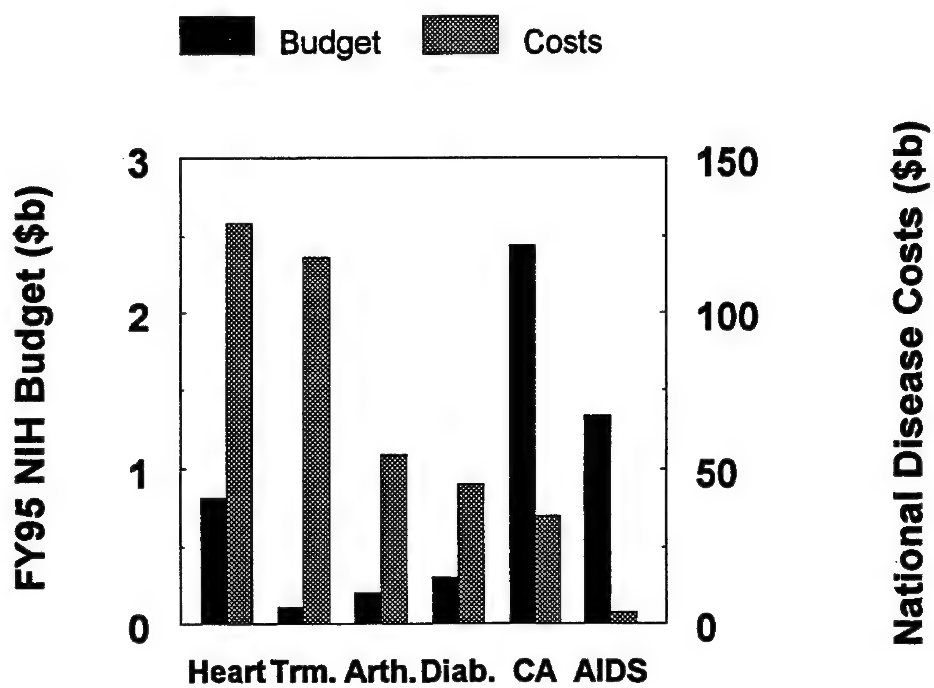
Dr. Constance Oliver

Thank you. Given the time, the program, and the schedule, I would very much like to thank both Anne and Thor for the excellent job they did in organizing the meeting. I know how much work it is. When a meeting runs smoothly it means that somebody's done a tremendous amount of work, so I'd like to thank them again for bringing us all together in what, for me, has been a very useful and very productive two days. I hope that all of you also found it useful. With that I'll turn it back to Thor. It's been a pleasure to meet some of you that I've only talked to on the telephone, and see some old friends again. Thank you.

Dr. Thor B. Nielsen

Thank you, Dr. Oliver. I'd like in particular to thank Dr. Murphy, without whose major efforts this symposium wouldn't have happened at all. I'd also like to thank Johanna Kidwell and Lisa Dalton for their tireless efforts. In a very real sense, I'd like to thank each and every one of you because a conference like this doesn't really work unless it affects the people who participate, unless those people make their own contribution, and it is reflected in their work. I hope that's been the case this time. Thank you very much. And perhaps we'll meet in another year.

Research Investment and Disease Costs



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